CHAPTER 2

ENZYMES OF $\Delta^1$-PYRROLINE-5-CARBOXYLATE METABOLISM
INTRODUCTION

Several metabolic roles have been suggested for arginase in animals which are deficient in urea cycle enzymes (Baby and Reddy, 1982). One possible role is that arginase provides ornithine for conversion into proline and glutamate through the intermediate formation of 4′-pyrroline-5-carboxylate (Fig. 0.1). Labelled arginine and ornithine give rise to labelled proline and glutamate in animal tissues (Eagle et al., 1965; Mepham and Linzell, 1967; Verbeke et al., 1968; Reddy and Campbell, 1969b; Clark et al., 1975; Mezl and Knox, 1977; Yoshida et al., 1977).

Three different enzymes are involved in the conversion of ornithine to proline and glutamate in vertebrate tissues. The first of these, ornithine aminotransferase (L-ornithine : 2-oxoacid aminotransferase, E.C.2.6.1.13) catalyzes the reversible transamination between L-ornithine and 2-oxoglutarate to form glutamic-γ-semialdehyde and glutamate (Quastel and Witty, 1951; Meister, 1954; Peraino and Pitot, 1963; Katunuma et al., 1964; Strecker, 1965; Herzfeld and Knox, 1968; Matsuzawa et al.,
Glutamic-\(\Delta\) semialdehyde is spontaneously converted to the cyclized form, \(\Delta'\)-pyrroline-5-carboxylate (Vogel and Davis, 1952; Fincham, 1953). The free energy of the ornithine aminotransferase reaction is highly unfavourable for ornithine formation (Strecker, 1965) and it has been concluded that this enzyme is primarily involved in ornithine catabolism (McGivan et al., 1977). \(\Delta'\)-Pyrroline-5-carboxylate is a key intermediate in the interconversions of ornithine, proline and glutamate (Fig. 0.1) and two unidirectional enzymes, \(\Delta\)-pyrroline-5-carboxylate reductase (L-proline-NAD(P)+-5-oxidoreductase, E.C.1.5.1.2; Smith and Greenberg, 1956, 1957; Meister et al., 1957; Adams and Goldstone, 1960a; Peisach and Strecker, 1962; Vallee et al., 1973) and \(\Delta'\)-pyrroline-5-carboxylate dehydrogenase (L-pyrroline-5-carboxylate-NAD\(^+\) oxidoreductase, E.C.1.5.1.12; Strecker, 1960; Adams and Goldstone, 1960b) catalyze its conversion to proline and glutamate respectively. A fourth enzyme proline oxidase, catalyzes the first step in L-proline catabolism and converts L-proline to \(\Delta'\)-pyrroline-5-carboxylate (Taggart and Krakaur, 1949; Johnson and Strecker, 1962; Kramar and Fitscha, 1970; Downing et al., 1977). The tissue
distribution, developmental changes and hormonal control of these enzymes, particularly ornithine aminotransferase, in mammals have drawn considerable attention (Peraino, 1968; Raiha and Kekomaki, 1968; Herzfeld and Greengard, 1968, 1969; Volpe et al., 1969; Rahman and Peraino, 1973; Morris and Peraino, 1976; Feraino et al., 1976; Herzfeld and Raper, 1976a,b; Kowaloff et al., 1976; Herzfeld et al., 1977).

Among the invertebrates the enzymes of 4'-pyrroline-5-carboxylate metabolism have been studied to some extent only in Tetrahymena (Hil1 and Chambers, 1967) and insects (Brosem and Veerabhadrapa, 1965; Reddy and Campbell, 1969b; Wadano et al., 1976; Yoshida et al., 1977; Pant and Kumar, 1978, 1979a; Tsuyama et al., 1978; Farmer et al., 1979).

The relative paucity of information on the enzymes of 4'-pyrroline-5-carboxylate metabolism in submammalian animals is due to the commercial non-availability of 4'-pyrroline-5-carboxylate as a substrate and the consequent necessity of synthesizing this compound by interested workers through elaborate chemical procedures (Vogel and Davis, 1952; Strecker, 1960b). The recent advent of simpler procedures for the synthesis of 4'-pyrroline-5-carboxylate by enzymatic (Smith et al., 1977) and chemical (Williams and Frank, 1975;
Mezl and Knox, 1976) methods has facilitated the studies on
the enzymes of \( \Delta^1 \)-pyrroline-5-carboxylate metabolism in
animals. Results on the distribution and preliminary
characterization of ornithine aminotransferase, \( \Delta^1 \)-pyrroline-
-5-carboxylate reductase, \( \Delta^1 \)-pyrroline-5-carboxylate
dehydrogenase and proline oxidase in tissue extracts of the
scorpion are presented here.

**MATERIALS AND METHODS**

**Chemicals**

Some of the important chemicals used in this study
were obtained as follows; sodium pyruvate and 2-oxo-
glutarate from British Drug Houses, oxaloacetic acid and
L-ornithine from Centron Research Laboratories (Bombay),
pyridoxal-5-phosphate and L-proline from E. Merck,
\( \delta \)-hydroxylysine-HCl (mixed DL and DL-allo), NADH and Dowex-50W
(4% cross-linked, dry mesh 20-50) from Sigma Chemical Co.,
St. Louis, Missouri, U.S.A. and Triton X-100 and NAD from the
Centre for Biochemicals, V.P. Chest Institute (Delhi).

**Preparation of \( \alpha \)-aminobenzaldehyde**

\( \alpha \)-Aminobenzaldehyde was prepared in the laboratory
from \( \alpha \)-nitrobenzaldehyde (Aldrich Chemicals Co., Milwaukee,
U.S.A.) as described by Albrecht, Scher and Vogel (1962).
o-Aminobenzaldehyde was dissolved in 40% (v/v) ethanol with gentle warming at 40°C and this alcoholic solution was used in the enzyme assays (Peraino and Pitot, 1963).

Preparation of DL-Δ⁻-pyrroline-5-carboxylate:

DL-Δ⁻-pyrroline-5-carboxylate (P-5-C) was prepared by the periodate oxidation of DL-Δ⁺-hydroxylysine as described by Williams and Frank (1975). One millimole of hydroxylysine was dissolved in 14 ml water in a brown bottle. To this solution at 4°C, 22 ml of a neutralized solution of 50 mM sodium metaperiodate was added. Eight minutes after mixing the two solutions, excess periodate was destroyed by adding 0.35 ml of 1 M glycerol. Two minutes later, the reaction mixture, held in ice, was acidified with 0.3 ml of 6 N HCl. The reaction mixture was chromatographed at 4°C on a column (2 x 31 cm) of Dowex-50, equilibrated with water after thorough washing with 1 N HCl. The sample adhering to the walls of the column was washed into the resin with 0.05 N HCl (10 ml). The column was eluted with 1.0 N HCl at room temperature (flow rate 50 ml/hr) and 7 ml fractions were collected. Δ⁺-Pyrroline-5-carboxylate was detected in the fractions by its reaction with o-aminobenzaldehyde. The absorbance was measured at 440 nm in a Toshniwal spectrophotometer and the concentration of Δ⁺-pyrroline-5-carboxylate was calculated by using the millimolar extinction coefficient.
**Fig. 2.0**

Elution profile of $\Delta'$-pyrroline-5-carboxylate (P-5-C) from the Dowex column. P-5-C in each fraction was determined by its reaction with o-aminobenzaldehyde.
Fig. 2.0

μmoles of P-5-C

Fraction Number
of 2.71 (Strecker, 1965). The yield of isolated $\Delta'$-pyrroline-5-carboxylate varied from 75 to 80% in five different preparations. The peak fractions (Fig. 2.0) of $\Delta'$-pyrroline-5-carboxylate were mixed and concentrated over potassium hydroxide and phosphorus pentoxide at $4^\circ$C in a vacuum desiccator. The resulting solution, which contains a racemic mixture of the D- and L-isomers of $\Delta'$-pyrroline-5-carboxylate, was adjusted to pH 6.8 with NaOH immediately before use. The exact concentration of $\Delta'$-pyrroline-5-carboxylate was determined in the neutralized solution by its reaction with o-aminobenzaldehyde and then used in the enzyme assays.

**Preparation of nucleotide solutions**

NADH solution used in $\Delta'$-pyrroline-5-carboxylate reductase assay was prepared fresh immediately before use in 0.05 M potassium phosphate buffer (pH 7.0). NAD$^+$ used in the $\Delta'$-pyrroline-5-carboxylate dehydrogenase assay was also prepared fresh before use.

**Assay of ornithine aminotransferase**

Tissues were homogenized in 9 volumes of chilled ($4^\circ$C) 0.02 M potassium phosphate buffer (pH 8.0) containing 0.14 M KCl (Hersfeld and Knox, 1963).

Ornithine aminotransferase activity was assayed in
tissue homogenates by the method of Peralno and Pitot (1963) with modifications. The reaction mixture in a total volume of 2.0 ml contained 70 μmoles of L-ornithine (pH 3.0), 20 μmoles of 2-oxoglutarate (pH 6.8), 100 μmoles of potassium phosphate buffer (pH 8.0), 0.11 μmole of pyridoxal-5-phosphate, 10 μmoles of o-aminobenzaldehyde and 0.2 ml of 10% (w/v) tissue homogenate. After incubation for 30 minutes at 37°C, the reaction was stopped by adding 1.0 ml of 10% TCA. In the control tubes, trichloroacetic acid was added to the reaction mixture before the addition of homogenate. The mixture was allowed to stand at room temperature for an additional 30 minutes to ensure complete colour development and filtered through Whatman No. 1 filter paper. The extinction of the filtrate was measured at 440 nm in a Beckman DU spectrophotometer. The amount of product, Δ′-pyrroline-5-carboxylate, formed was calculated from the millimolar extinction coefficient (2.71) of this compound reported by Strecker (1965). The enzyme activity was expressed as μmoles of product formed per hour per gram of tissue or per mg protein.

Molecular weight of ornithine aminotransferase

Molecular weight of ornithine aminotransferase in hepatopancreas of scorpion was determined by gel filtration
on Sephadex G-100 (Andrews, 1965). The gel was allowed to swell in distilled water and fine particles which float on top were decanted. The gel was packed at 4°C in a column (2.2 x 26 cm) and equilibrated with 0.05 M tris-Cl buffer (pH 7.5) containing 0.1 M KCl. The column was calibrated with rabbit muscle aldolase (mol.wt. 150,000; Hasse, 1964), bovine serum albumin (mol.wt. 67,000), ovalbumin (mol.wt. 46,000) and horse heart cytochrome c (mol.wt. 12,400) as described for Sephadex G-200 in the previous chapter on urea cycle enzymes (p. 25-26). The void volume of the column determined by Dextran blue was 40 ml.

40% (w/v) homogenates of tissues were prepared in 0.05 M tris-chloride buffer (pH 7.5) containing 0.1 M KCl. Homogenates were centrifuged at 4°C and 5,000 rpm for 15 minutes and the supernatants (2.0 ml) were loaded on the gel column. 50% (w/v) homogenate of rat liver was prepared in distilled water and centrifuged at 5,000 rpm and 4°C for 15 minutes. Equal volumes of the supernatant and 0.1 M tris-chloride buffer (pH 7.5) containing 0.2 M KCl were mixed. This diluted extract (2 ml) was loaded on the column. Fractions (2.0 ml) were collected in both cases manually and 0.9 ml aliquots of fractions were assayed for ornithine aminotransferase as described above.
Subcellular distribution of ornithine aminotransferase:

Scorpion hepatopancreas and rat liver tissues were homogenized in 0.05 M potassium phosphate buffer (pH 8.0) containing 0.25 M sucrose and centrifuged at 500 g and 4°C for 15 minutes. The supernatant was decanted and centrifuged at 15,000 g and 4°C for 15 minutes. The 500 g and 15,000 g residues were resuspended in the original volume of the homogenizing medium and assayed, along with the whole homogenate and 15,000 g supernatant, for ornithine aminotransferase activity as described above.

Assay of \( \Delta^1 \)-pyrroline-5-carboxylate reductase:

Homogenates (10% w/v) of scorpion tissues were prepared in 0.05 M potassium phosphate buffer (pH 6.3) containing 0.25 M sucrose and 2.5 mM mercaptoethanol (Herzfeld et al., 1977). Since \( \Delta^1 \)-pyrroline-5-carboxylate reductase is known to be cold sensitive (Herzfeld and Raper, 1976b, Herzfeld et al., 1977), homogenates were prepared at room temperature.

The activity of the enzyme in whole homogenates was assayed by the method of Herzfeld et al. (1977) with modifications. The reaction mixture consisted of 53.0 \( \mu \)moles of potassium phosphate buffer (pH 7.2), 4 to 8 \( \mu \)moles of
DL-$\Delta'$-pyrroline-5-carboxylate (pH 6.8), 3.0 $\mu$moles of NADH and tissue extracts in a total volume of 1.0 ml. The reaction mixture was equilibrated to $37^\circ$C before addition of enzyme. NADH was omitted from the control tubes. After incubation at $37^\circ$C for 10 minutes the reaction was stopped by adding 1.0 ml of 10% trichloroacetic acid. The precipitated protein was centrifuged and proline in the supernatant was estimated after nitrosation by the acid ninhydrin method according to Herzfeld et al. (1977).

$\Delta'$-Pyrroline-5-carboxylate dehydrogenase assay:

Tissues were homogenized in 9 volumes of ice-cold 0.05 M potassium phosphate buffer (pH 6.8) containing 0.25 M sucrose and 0.5% Triton X-100 (Herzfeld et al., 1977). The homogenates were centrifuged at 20,000 g and $4^\circ$C for 20 minutes. The supernatants were used in enzyme assays after suitable dilution.

The activity of this enzyme was measured by the method of Herzfeld et al. (1977) with minor modifications. Reaction mixture contained 90 $\mu$moles of potassium phosphate buffer (pH 7.8), 0.6 $\mu$moles of DL-$\Delta'$-pyrroline-5-carboxylate (pH 6.8), 4 $\mu$moles of $NAD^+$ and 0.05 ml of 10% (w/v) homogenate in a total volume of 1.0 ml. The reaction was initiated by adding NAD. The formation of NADH at room temperature
(30 ± 1°C) in 5 minutes was monitored at 340 nm in a u.v.
spectrophotometer (Toshniwal Bros., India). Substrate free
blank activities were subtracted from the experimental
values. NADH formed in the reaction was calculated from the
millimolar extinction coefficient of 6.22 (Plummer, 1978).

Assay of proline oxidase:

Tissues were homogenized in 9 volumes of 0.1 M
potassium phosphate buffer (pH 7.5) containing 10 mM EDTA
(Brosemer and Veerabhadrappa, 1965). The homogenate was
centrifuged at 20,000 g and 4°C for 15 minutes. Supernatant
was discarded and residue was resuspended in the original
volume of homogenizing medium and assayed for proline oxidase
activity by the method of Herzfeld et al. (1977). The
reaction mixtures containing 132 mM potassium phosphate
buffer (pH 7.5), 158 mM L-proline, 1.6 mM cytochrome c and 0.5
ml enzyme extract in a total volume of 1.9 ml were incubated
at 37°C with shaking. The reaction was stopped after
30 minutes by adding 1.0 ml of 10% trichloroacetic acid. The
\( \Delta^1 \)-pyrroline-5-carboxylate formed was determined by the
addition of 0.1 ml of 0.2 M o-aminobenzaldehyde. Tubes were
kept at room temperature for 30 minutes for complete colour
development. The solution was filtered through Whatman No. 1
filter paper and the absorbance was measured at 440 nm in a
spectrophotometer (Toshniwal Bros., India). The amount of
product $\Delta^1$-pyrroline-5-carboxylate formed was calculated from the millimolar extinction coefficient (2.71) reported by Strecker (1965). The enzyme activity was expressed as μmoles of $\Delta^1$-pyrroline-carboxylate formed per hour per gram of tissue or per mg protein. Minus-proline controls were employed.

RESULTS

**Ornithine aminotransferase**:

The ornithine aminotransferase assay showed absolute dependence on the presence of the substrates, L-ornithine and 2-oxoglutarate. The enzyme activity was almost totally abolished by boiling the homogenate (Table 2.1). Deletion of pyridoxal-5-phosphate from the assay mixture resulted in the loss of about 96% activity. Thus pyridoxal-5-phosphate is required as a coenzyme for catalytic activity of the enzyme. Mammalian ornithine aminotransferase is known to bind 4 moles of pyridoxal-5-phosphate per mole of enzyme (Jenkins and Tsai, 1970; Morris et al., 1974; Kalita et al., 1976). There is also evidence for the formation of pyridoxamine-enzyme complex as an intermediate in the reaction (Peraino, 1972) and for the specific role of pyridoxal-5-phosphate in maintaining the appropriate conformational state of the enzyme (Sanada et al., 1976).
Effect of omitting the assay system components on ornithine aminotransferase activity in scorpion hepatopancreas.

0.2 ml of 10% homogenate was incubated for 30 minutes in the standard assay system and L-pyrroline-5-carboxylate formed was measured. The relative activities are shown in parentheses.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>µmoles/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.140 (100)</td>
</tr>
<tr>
<td>Minus-pyridoxal-5-phosphate</td>
<td>0.006 (4.3)</td>
</tr>
<tr>
<td>Minus L-ornithine</td>
<td>0.000 (0)</td>
</tr>
<tr>
<td>Minus 2-oxoglutarate</td>
<td>0.003 (2.1)</td>
</tr>
<tr>
<td>Minus homogenate</td>
<td>0.000 (0)</td>
</tr>
<tr>
<td>Minus o-aminobenzaldehyde</td>
<td>0.000 (0)</td>
</tr>
<tr>
<td>Complete but heat inactivated enzyme</td>
<td>0.006 (4.3)</td>
</tr>
</tbody>
</table>
The formation of pyrroline-5-carboxylate under the assay conditions was linear up to sixty minutes of incubation and 0.4 ml of 10% (w/v) homogenate (Fig. 2.1).

Ornithine aminotransferase activity in hepatopancreas of scorpion had a pH optimum of 8.0 in phosphate buffer and 7.3 in tris-chloride buffer (Fig. 2.2). The pH optima reported for this enzyme from other sources varied between 7.4 and 8.0 (Meister, 1954; Vogel and Kopac, 1960; Persino and Pitot, 1963; Katumma et al., 1964; Vecchio and Kalman, 1968; Reddy and Campbell, 1969b; Sanada et al., 1970; Jenkins and Tsai, 1970; Wekell and Brown, 1973).

Ornithine aminotransferase from the hepatopancreas of scorpion was highly specific to 2-oxoglutarate as the amino acceptor (Table 2.2). Freshly prepared solutions of pyruvate and oxaloacetate were only about 6-7% as effective as 2-oxoglutarate. Although Quastel and Witty (1951), and Meister (1954) have initially reported that pyruvate is a better amino acceptor in ornithine aminotransferase reaction, many workers have subsequently demonstrated that 2-oxoglutarate is the most active amino acceptor for the enzyme from many sources (Vogel and Kopac, 1960; Katumma et al., 1964; Hill and Chambers, 1967; Vecchio and Kalman, 1968; Reddy and Campbell, 1969b; Jenkins and Tsai, 1970; Sanada et al., 1970;
Effect of enzyme concentration (A) and incubation time (B) on ornithine aminotransferase activity of scorpion hepatopancreas. Different volumes of a 10% (w/v) homogenate (0.05 ml, 0.1 ml, 0.2 ml, 0.3 ml and 0.4 ml corresponding to 2.04 mg, 4.08 mg, 8.16 mg, 12.24 mg and 16.32 mg of protein) were incubated for 30 minutes. Similarly 0.1 ml of a 10% (w/v) homogenate was incubated for different times and in both cases Δ-pyrroline-5-carboxylate formed in the reaction was measured.
Effect of pH on ornithine aminotransferase activity.  
0.1 ml of 10% (w/v) homogenate of scorpion hepatopancreas was incubated for 30 minutes in a reaction mixture containing (50 mM) potassium phosphate buffer (open circles) or tris-chloride buffer (closed circles) of different pH. The pH values indicated represent those measured in the reaction mixtures during the first 15 minutes of incubation.
Fig. 2.2

![Graph showing the relationship between pH and μ moles/hr/mg protein. The graph has two lines, one with filled circles and one with open circles. The pH range is 6.0 to 9.0, and the μ moles/hr/mg protein range is 0.02 to 0.14.](image-url)
2-Oxoadid specificity of ornithine aminotransferase in scorpion hepatopancreas.

2-Oxoglutarate in the standard assay system was replaced by freshly prepared solutions of pyruvate and oxaloacetate (10 mM, pH 6.8) and \( \Delta^1 \)-pyrroline-5-carboxylate formed was measured. The relative activities with the three oxoacids are given in parentheses.

<table>
<thead>
<tr>
<th>2-Oxoadid</th>
<th>( \mu )moles/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Oxoglutarate</td>
<td>0.153 (100)</td>
</tr>
<tr>
<td>( \alpha )-Oxaloacetate</td>
<td>0.011 (7.0)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.009 (5.7)</td>
</tr>
</tbody>
</table>
Thus, as pointed out by Strecker (1965), 2-oxoglutarate is probably the physiological substrate for the enzyme.

Fig. 2.3 shows the enzyme activity as a function of varying ornithine concentration at a fixed concentration of 2-oxoglutarate (10 mM). The ornithine concentration routinely used in the assays (35 mM) provided the saturating concentration for the enzyme (Fig. 2.3). The apparent Km values of the enzyme calculated for L-ornithine from double reciprocal plots in three experiments were 2.0, 2.3 and 5.3 mM.

Fig. 2.4 provides similar data on enzyme activity at different concentrations of 2-oxoglutarate and a fixed concentration of L-ornithine (35 mM). The 10 mM 2-oxoglutarate concentration used in the standard incubation mixture saturated the enzyme. The apparent Km of the enzyme for 2-oxoglutarate determined from double reciprocal plots was 1.6 mM.

The Km values of scorpion ornithine aminotransferase are similar to those reported for the enzyme from rat tissues (Percino and Pitot, 1963; Herzfeld and Knox, 1968; Herzfeld and Raper, 1976b), chicken liver (Vecchio and Kalman, 1963), trout liver (Wekell and Brown, 1973), insect fat body
Effect of substrate concentration (L-ornithine) on ornithine aminotransferase activity. The enzyme activity (V) was assayed at different concentrations of L-ornithine (S). The 2-oxoglutarate concentration was kept constant (10 mM). S versus V plot is shown in Fig. A and Lineweaver-Burk double reciprocal plot in Fig. B.
Ornithine aminotransferase activity (V) in scorpion hepatopancreas as a function of 2-oxoglutarate concentration (S). L-ornithine concentration was kept constant (35 mM). S versus V plot is shown in Fig. A and Lineweaver-Burk double reciprocal plot in Fig. B.
Fig. 24

\[ \frac{1}{V} \left( \frac{\mu \text{ moles/hr/mg protein}}{1} \right) \]

\[ \alpha \text{-Ketoglutarate (mM)} \]

\[ \frac{\alpha \text{-Ketoglutarate (mM)}}{1.0} \]
(Reddy and Campbell, 1969b), *Tetrahymena* (Hill and Chambers, 1967) and *Neurospora* (Vogel and Kopac, 1960). Further more ornithine aminotransferase from rat liver (Strecker, 1965), rat kidney (Yip and Collins, 1971), trout liver (Wekell and Brown, 1973) and insect fat body (Reddy and Campbell, 1969b) is known to be inhibited by one or both of the substrates. No such inhibition of scorpion enzyme by L-ornithine and 2-oxoglutarate was noticed.

Fig. 2.5 shows the enzyme activity as a function of varying pyridoxal-5-phosphate concentration in the presence of 35 mM ornithine and 10 mM 2-oxoglutarate. The concentration of pyridoxal-5-phosphate (0.05 mM) used in the standard assay system was adequate to saturate the enzyme.

Fig. 2.6 shows the elution profiles of ornithine aminotransferase from scorpion hepatopancreas and rat liver through a column of Sephadex G-100. Both enzymes eluted out as single symmetrical peaks. From the calibration curve shown in Fig. 2.7, ornithine aminotransferase from scorpion hepatopancreas and rat liver gave molecular weights of 80,000 and 72,000 respectively. On a column of Sephadex G-200, scorpion enzyme yielded a mol.wt. of 70,000.

The molecular weight (70,000-80,000) determined here for scorpion hepatopancreatic and rat liver ornithine
Ornithine aminotransferase activity of scorpion hepatopancreas at different concentrations of pyridoxal-5-phosphate.
Fig. 2.5

![Graph showing the relationship between Pyridoxal-5-phosphate concentration (mM) and the rate of reaction (μ moles/hr/mg protein).]
Gel filtration profiles of ornithine amino-transferase from scorpion hepatopancreas (open circles and continuous line) and rat liver (closed circles and broken line) on Sephadex G-100. The fraction containing highest activity was arbitrarily assigned a value of 100 in both cases and the activity in other fractions was plotted relative to this. The elution volumes of dextran blue and proteins of known molecular weight are indicated by arrows.
Relative activity

Elution Volume (ml)

20 40 60 80 100

Dextran Blue
Aldolase
Serum Albumin
Ovalbumin
Cytochrome c

Fig 2.6
Calibration of the Sephadex G-100 column for molecular weight determination of ornithine aminotransferase from scorpion hepatopancreas (SOAT) and rat liver (ROAT). The proteins used for calibration of the column were cytochrome c (1), ovalbumin (2), bovine serum albumin (3) and rabbit muscle aldolase (4).
aminotransferase is only about half of the molecular weight (132,000-180,000) reported in the literature for the rat liver enzyme (Matsuzawa et al., 1968; Peraino et al., 1969; Kalita et al., 1976; Sanada et al., 1976). Mammalian ornithine aminotransferase is a tetramer with a subunit molecular weight of 33,000-45,000 (Peraino et al., 1969; Kalita et al., 1976; Sanada et al., 1976). All these reports on the molecular weight of mammalian ornithine aminotransferase are based on studies with purified enzyme preparations, while crude tissue extracts have been employed in the present study for molecular weight determination.

Morris et al. (1974) have reported that the gel filtration behaviour of purified ornithine aminotransferase is different from that of the enzyme in crude extracts. According to them the enzyme exists as a dimer in crude extracts and dilute solutions, but aggregates to yield the tetramer with increasing concentration of the pure enzyme. Boernke et al. (1981) also concluded that molecular weight of ornithine aminotransferase is concentration dependent and that the enzyme aggregates as its concentration increases. Thus it appears that the enzyme exists as a dimer in nature and the tetramer results from the aggregation of the dimers during purification.

Ornithine aminotransferase activity could not be
detected in the pedipalp muscle and telson of the scorpion (Table 2.3).

Subcellular distribution of ornithine aminotransferase in scorpion hepatopancreas and rat liver is shown in Table 2.4. In scorpion hepatopancreas, the enzyme was found to be largely localized in the post-mitochondrial supernatant (15,000 g supernatant). However, a major portion of this enzyme activity in rat liver was associated with the mitochondrial fraction (15,000 g residue) in agreement with the earlier reports that it is a mitochondrial enzyme in mammalian tissues (Swick et al., 1970; McGivan et al., 1977) and blowfly fat body (Tsuyama et al., 1973). Ornithine aminotransferase has been shown to be a soluble enzyme in Neurospora (Vogel and Kopac, 1960) and silkworm fat body (Reddy and Campbell, 1969b).

\[ \Delta^1\text{-pyrroline-5-carboxylate reductase} \]

Under the assay conditions employed for scorpion hepatopancreatic \( \Delta^1\text{-pyrroline-5-carboxylate reductase} \), the product formation was linear up to 12 minutes with respect to incubation time and up to 0.20 ml (10%, w/v) homogenate with respect to enzyme concentration (Fig. 2.8). Proline formation in the absence of added substrates was only about 25% of that in the presence of the substrates. In all experiments, minus-substrate controls were employed.
Table 2.3

Distribution of ornithine aminotransferase activity in scorpion tissues.

Values given are means ± standard deviations with ranges in parentheses.

<table>
<thead>
<tr>
<th>Tissues (No. of samples)</th>
<th>μmoles/hr/gm (Range)</th>
<th>μmoles/hr/mg protein (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas (9)</td>
<td>40 ± 18 (12 - 68)</td>
<td>0.14 ± 0.02 (0.02 - 0.32)</td>
</tr>
<tr>
<td>Pedipalp muscles</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>Telson</td>
<td>BLD</td>
<td>BLD</td>
</tr>
</tbody>
</table>

BLD : Below the level of detection.
### Table 2.4

Subcellular distribution of ornithine aminotransferase activity in scorpion hepatopancreas and rat liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Distribution of activity</th>
<th>Scorpion hepatopancreas</th>
<th>Rat liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>500 g residue</td>
<td>5</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>15,000 g residue</td>
<td>5</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>15,000 g supernatant</td>
<td>94</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
Scorpion hepatopancreas \( \Delta^-\)pyrroline-5-carboxylate reductase activity in relation to enzyme concentration (A) and incubation time (B). Different volumes of a 10% (w/v) homogenate (0.05 ml, 0.1 ml, 0.15 ml and 0.2 ml corresponding to 1.7, 3.4, 5.1 and 6.8 mg protein respectively) were incubated for 10 minutes. Similarly 0.1 ml of 10% (w/v) homogenate was incubated for 3, 6, 9 and 12 minutes, at 37°C. In both cases, proline formed was measured.
The pH optimum of \( \Delta^1 \)-pyrroline-5-carboxylate reductase in scorpion hepatopancreas was about 6.5 (Fig. 2.9), which is similar to that reported for the enzyme in mammalian liver (Smith and Greenberg, 1957; Meister et al., 1957; Peisach and Strecker, 1962) insects (Reddy and Campbell, 1969a; Wadano et al., 1976) and \( \text{E. coli} \) (Rossi et al., 1977).

The concentrations of \( \Delta^1 \)-pyrroline-5-carboxylate (1 to 8 mM) and NADH (3 mM) employed in the assays routinely provided nearly the saturating concentration (Figs. 2.10 and 2.11). There was no inhibition of the enzyme when the concentrations of \( \Delta^1 \)-pyrroline-5-carboxylate and NADH were increased to 13 mM and 6 mM respectively (Figs. 2.10 and 2.11). The apparent \( K_m \) values of the enzyme calculated for \( \Delta^1 \)-pyrroline-5-carboxylate and NADH from double reciprocal plots were 0.53 mM and 0.33 mM respectively. The reductase is specific only for the L-isomer of \( \Delta^1 \)-pyrroline-5-carboxylate (Meister et al., 1957; Peisach and Strecker, 1962; Williams and Frank, 1975). Consequently only the concentration of L-pyrroline-5-carboxylate was considered in the calculations of the \( K_m \), although an equal quantity of the D-isomer was also present in the reaction mixture. The apparent \( K_m \) values of the scorpion enzyme determined here are of the same order as those reported for the mammalian
Effect of pH on scorpion hepatopancreas 
\( \Lambda^1 \text{-pyrroline-5-carboxylate reductase activity.} \)
0.1 mL of 10% homogenate was incubated at 37°C in reaction mixtures containing 53 mM potassium phosphate buffers of different pH. The pH values indicated represent those measured in the complete reaction mixtures during incubation.
Fig. 2.9

\[ \text{μ moles/hr/mg protein} \]

\[ \text{pH} \]
Effect of substrate concentration (DL-Δ¹-pyrroline-5-carboxylate) on pyrroline-5-carboxylate reductase activity in scorpion hepatopancreas. The enzyme activity (V) was assayed at different concentrations of DL-Δ¹-pyrroline-5-carboxylate (S) but at a fixed concentration of NADH (3 mM). S versus V plot is shown in Fig. A and Lineweaver-Burk double reciprocal plot in Fig. B.
Effect of NADH concentration on D-\text{pyrroline}-5-carboxylate reductase in scorpion hepatopancreas. The enzyme activity (V) was assayed at different concentrations of NADH (S) and a fixed concentration of DL-D-\text{pyrroline}-5-carboxylate (200 mM). S versus V plot is shown in Fig. A and Lineweaver-Burk double reciprocal plot in Fig. B.
(Smith and Greenberg, 1957; Vallee et al., 1973; Peisach and Strecker, 1962; Herzfeld et al., 1977), silkmoth (Reddy and Campbell, 1969b) and blowfly (Wadano et al., 1976) enzymes, but higher than those reported by Farmer et al. (1979) for the Drosophila enzyme.

The activities of Δ¹-pyrroline-5-carboxylate reductase assayed in scorpion tissues under conditions found optimal for hepatopancreas are given in Table 2.6. Highest activity was found in hepatopancreas and lowest in the telson with intermediate activity in the pedipalp muscles.

Δ¹-Pyrroline-5-carboxylate dehydrogenase

Reduction of NAD by extracts of scorpion hepatopancreas in the presence of Δ¹-pyrroline-5-carboxylate was linear up to at least four minutes of incubation (Fig. 2.12). Similarly, NADH formation increased linearly with enzyme concentration up to 0.05 ml of 10% (w/v) homogenate (Fig. 2.12). On boiling, the extracts totally lost their ability to reduce NAD in the presence of Δ¹-pyrroline-5-carboxylate (Table 2.7). Deletion of Δ¹-pyrroline-5-carboxylate from the reaction mixtures still yielded 37% as much reduction of NAD as in its presence. Consequently, Δ¹-pyrroline-5-carboxylate-minus controls were routinely employed in the assays to correct for this non-specific reduction. There was no
Effect of omission of the assay system components on Δ⁴-pyrroline-5-carboxylate reductase activity in scorpion hepatopancreas.

0.1 ml of 10\% (w/v) homogenate was incubated for 10 minutes in the standard assay system and proline formed was measured. Relative activities are shown in parentheses.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>μmoles proline/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.01 (100)</td>
</tr>
<tr>
<td>Minus NADH</td>
<td>0.28 (28.1)</td>
</tr>
<tr>
<td>Minus Δ⁴-pyrroline-5-carboxylate</td>
<td>0.21 (21.1)</td>
</tr>
<tr>
<td>Minus homogenate</td>
<td>0.07 (7.0)</td>
</tr>
<tr>
<td>Complete but boiled homogenate</td>
<td>0.18 (17.5)</td>
</tr>
</tbody>
</table>
Distribution of Δ-pyrroline-5-carboxylate reductase activity in scorpion tissues.

Values given are means ± standard deviations with ranges in parentheses.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>µmoles proline/hr/gm</th>
<th>µmoles proline/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>(No. of samples)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatopancreas (8)</td>
<td>302 ± 62</td>
<td>1.23 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>(204 - 384)</td>
<td>(0.86 - 1.75)</td>
</tr>
<tr>
<td>Pedipalp muscles (5)</td>
<td>35 ± 14</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(18 - 48)</td>
<td>(0.25 - 0.53)</td>
</tr>
<tr>
<td>Telson (5)</td>
<td>10.8 ± 0.06</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(18 - 6)</td>
<td>(0.22 - 0.13)</td>
</tr>
</tbody>
</table>
Δ'-Pyrroline-5-carboxylate dehydrogenase activity in scorpion hepatopancreas as a function of enzyme concentration (A) and incubation time (B).

Different volumes of 10% homogenate (0.01, 0.02, 0.03, 0.04 and 0.05 ml corresponding to 0.14, 0.28, 0.42, 0.56 and 0.70 mg protein respectively) were incubated for 5 minutes. Similarly 0.05 ml of 10% homogenate was incubated for different times. In both cases, NADH formation was measured at 30°C by monitoring the increase in absorbance at 340 nm.
Table 2.7

Effect of omission of the assay system components on 1-oxo-4-carboxylate dehydrogenase activity in scorpion hepatopancreas.

0.05 ml of 10% homogenate was incubated with the standard assay system and the reduction of NAD was measured at 340 nm. Relative activities are shown in parentheses.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>μmoles of NADH/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.082 (100)</td>
</tr>
<tr>
<td>Minus 1-oxo-4-carboxylate</td>
<td>0.031 (37)</td>
</tr>
<tr>
<td>Minus NAD</td>
<td>0.020 (25)</td>
</tr>
<tr>
<td>Minus homogenate</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>Complete but boiled homogenate</td>
<td>0.00 (0)</td>
</tr>
</tbody>
</table>
reduction of NAD in the absence of enzyme. P-5-C dehydrogenase from scorpion hepatopancreas showed maximum activity around pH 7.8 (Fig. 2.13) which is similar to the pH optimum reported by Adams and Goldstone (1960b) and Herzfeld et al. (1977) for the enzyme in calf and rat liver. Strecker (1960e) however, reported a more alkaline pH optimum (8.5-8.6) for ox liver \(\Delta^1\)-pyrroline-5-carboxylate dehydrogenase.

The substrate saturation curve of \(\Delta^1\)-pyrroline-5-carboxylate dehydrogenase for \(\Delta^1\)-pyrroline-5-carboxylate is shown in Fig. 2.14. 0.6 mM DL-\(\Delta^1\)-pyrroline-5-carboxylate routinely used in the assays provided the optimal substrate concentration where the activity measured is the highest. The activity was strongly inhibited at higher concentrations of this substrate. The activity recorded at 0.62 mM pyrroline-5-carboxylate concentration was about 55% of the theoretical \(V_{\text{max}}\). Strecker (1960a) also reported inhibition of the ox liver enzyme at high concentrations of \(\Delta^1\)-pyrroline-5-carboxylate. Concentration of NAD (4 mM) used in the standard assay of \(\Delta^1\)-pyrroline-5-carboxylate dehydrogenase in scorpion hepatopancreas was also a saturating concentration (Fig. 2.15).

The apparent \(K_m\) values of scorpion hepatopancreatic \(\Delta^1\)-pyrroline-5-carboxylate dehydrogenase calculated from
Effect of pH on the activity of Δ³-pyrroline-5-carboxylate dehydrogenase in scorpion hepatopancreas. 0.05 ml of 10% (w/v) homogenate was incubated for 5 minutes in the reaction mixtures containing 90 mM potassium phosphate buffers of different pH. The pH values indicated, represent those measured in the reaction mixtures at the end of incubation.
Fig. 2.13

[Graph showing the relationship between pH and μmoles/hr/mg protein with data points at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5]
Effect of DL-\(\Delta^1\)-pyrroline-5-carboxylate (P-5-C) concentration on \(\Delta^1\)-pyrroline-5-carboxylate dehydrogenase activity in scorpion hepatopancreas. P-5-C concentration (S) was varied keeping the NAD concentration constant (4 mM) and NAD reduction was measured (V). S versus V plot is shown in Fig. A and Lineweaver-Burk double reciprocal plot in Fig. B.
Fig 214

\[ \text{P-5-C (mM)} \]

\[ \frac{1}{V} (\mu \text{ moles/hr/mg protein})^{-1} \]

\[ \frac{1}{P5-C (\text{mM})} \]
Effect of NAD concentration (S) on \( \Delta^1 \)-pyrroline-5-carboxylate dehydrogenase (V) of scorpion hepatopancreas. NAD concentration was varied keeping the DL-\( \Delta^1 \)-pyrroline-5-carboxylate concentration constant (0.6 mM). The NAD concentration (S) versus NADH production (V) is shown in Fig. A. Reciprocal plot is shown in Fig. B.
double reciprocal plots (Figs. 2.14 and 2.15) were 0.14 mM and 0.36 mM for L-pyrroline-5-carboxylate and 0.87 mM for NAD. Although Δ′-pyrroline-5-carboxylate dehydrogenase from ox liver (Strecker, 1960a) and insect flight muscle (Brosemer and Veerabhadrapa, 1965) were earlier thought to be non-stereospecific, Herzfeld et al. (1977) have recently demonstrated the extreme specificity of the rat and calf liver enzymes to the L-isomer of Δ′-pyrroline-5-carboxylate. Therefore, as in the case of Δ′-pyrroline-5-carboxylate reductase, only the concentration of L-Δ′-pyrroline-5-carboxylate was taken into account while calculating the Kn. The apparent Kn values of the scorpion enzyme determined here for L-Δ′-pyrroline-5-carboxylate and NAD are comparable to those of the enzyme from mammalian liver (Strecker, 1960a; Herzfeld et al., 1977).

The activity of Δ′-pyrroline-5-carboxylate dehydrogenase in the hepatopancreas and pedipalp muscles of the scorpion is shown in Table 2.8. No activity of this enzyme could be detected in the telson.

**Proline oxidase:**

Proline oxidase in scorpion muscle, like in rat liver, is exclusively localized in the 20,000 g residue (Table 2.9). Thus, proline oxidase in scorpion muscle
Distribution of $\Delta^\text{I}$-pyrroline-5-carboxylate dehydrogenase activity in scorpion tissues.

Values given are means ± standard deviations with ranges in parentheses.

<table>
<thead>
<tr>
<th>Tissues (No. of observations)</th>
<th>μmoles/hr/gm</th>
<th>μmoles/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas (11)</td>
<td>26 ± 6</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(19 - 39)</td>
<td>(0.17 - 0.37)</td>
</tr>
<tr>
<td>Pedipalp muscles (6)</td>
<td>8 ± 4</td>
<td>0.26 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>(4 - 10)</td>
<td>(0.14 - 0.43)</td>
</tr>
<tr>
<td>Telson</td>
<td>BLD</td>
<td>BLD</td>
</tr>
</tbody>
</table>

BLD : Below the level of detection.
Table 2.9

Assay of proline oxidase in fractions of scorpion muscle and rat liver homogenates.

Relative activities are shown in parentheses.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Scorpion muscle</th>
<th>Rat Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>5.2 (100)</td>
<td>16.1 (100)</td>
</tr>
<tr>
<td>20,000 g residue</td>
<td>5.4 (104)</td>
<td>19.9 (124)</td>
</tr>
<tr>
<td>10,000 g supernatant</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
</tr>
</tbody>
</table>
appears to be a mitochondrial enzyme as in rat liver
(Brunner and Neupert, 1969) and insect tissues (Brosemer and
Veerabhadrappa, 1965; Yoshida et al., 1977). In the proline
oxidase assay in extracts of scorpion pedipalp muscles,
N'-pyrroline-5-carboxylate formation was linear up to
40 minutes of incubation (Fig. 2.16). Similarly the product
formation increased linearly with enzyme concentration up to
0.3 ml of 10% (w/v) homogenate (Fig. 2.16). Cytochrome c
stimulated the proline oxidase activity considerably
(Table 2.10). Deletion of proline and enzyme from the
reaction mixture or inclusion of boiled homogenate still
gave 20% as much activity as complete reaction mixture.
Hence minus-enzyme and minus-proline controls were always
employed in the assays to correct for this non-specific
activity. The optimum pH for proline oxidase activity in
scorpion pedipalp muscle preparations was 7.5 (Fig. 2.17).
The pH optima for proline oxidase in rat liver (Herzfeld et al.,
1977) and insect tissues (Brosemer and Veerabhadrappa, 1965;
Yoshida et al., 1977) were reported to vary from 7.0 to 8.0.

The 158 mM proline concentration routinely employed
in the assays was sufficient to saturate the enzyme under
the assay conditions and the apparent Km values of the
enzyme for L-proline calculated from the double reciprocal
plots in two experiments were 3.8 mM and 4.4 mM (Fig. 2.18).
Effect of enzyme concentration (A) and incubation time (B) on proline oxidase activity of scorpion pedipalp muscles. Different volumes of 10% (w/v) homogenate (0.15, 0.30, 0.5 and 0.7 ml corresponding to 2.25, 4.5, 7.5 and 10.5 mg of proteins respectively) were incubated for 30 minutes. Similarly 0.5 ml of 10% (w/v) homogenate of scorpion pedipalp muscles was incubated at 37°C for different periods with constant shaking. In both cases, Δ-pyrroline-5-carboxylate formed was measured.
Fig 2.16

A. 

B.
Table 2.10

Effect of omitting the assay system components on proline oxidase activity in preparations of scorpion pedipalp muscles.

0.5 ml of 20,000 g residue suspension was incubated for 30 minutes in the standard assay system and Δ^-pyrroline^-5-carboxylate formed was measured. Relative activities are shown in parentheses.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>μmoles/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.10 (100)</td>
</tr>
<tr>
<td>Minus proline</td>
<td>0.02 (20)</td>
</tr>
<tr>
<td>Minus cytochrome c</td>
<td>0.03 (33)</td>
</tr>
<tr>
<td>Minus homogenate</td>
<td>0.02 (20)</td>
</tr>
<tr>
<td>Complete but heat inactivated enzyme</td>
<td>0.02 (20)</td>
</tr>
</tbody>
</table>
Effect of pH on proline oxidase activity in scorpion pedipalp muscle. 0.5 ml of 10% (w/v) 
homogenate of scorpion pedipalp muscles was incubated at 37°C for 30 minutes with constant 
shaking. The reaction mixture contained 132 mM potassium phosphate buffers of different pH. 
The pH values indicated represent those measured in the complete reaction mixtures during the 
first 10 minutes of incubation.
Fig. 2.17

![Graph showing pH vs. $\mu$ moles/hr/mg protein](image)

- pH scale: 6.5 - 8.5
- $\mu$ moles/hr/mg protein scale: 0.005 - 0.030

The graph illustrates the relationship between pH and the activity of a particular enzyme, with the peak activity occurring at pH 7.5.
Effect of substrate concentration (L-proline) on proline oxidase activity in scorpion muscle. The proline oxidase activity (V) was assayed in the presence of different concentrations of L-proline (S) at a fixed concentration of cytochrome c (1.6 mIU). S versus V plot is shown in Fig. A and Lineweaver-Burk double reciprocal plot in Fig. B.
Fig. 2.18

A

B

$\frac{1}{V} (\mu \text{ moles/hour/mg protein})^{-1}$
Proline oxidase from *Locusta migratoria* (Brosemmer and Veerabhadrappa, 1965) and *Aldrichina arahela* (Yoshida et al., 1977) gave half maximal velocity at 15 mM and 8.3 mM L-proline concentrations respectively. However, mammalian proline oxidase has a lower Km for L-proline (Kramar and Fitscha, 1970; Kovaloff et al., 1976; Downing et al., 1977). Like the insect enzyme (Brosemmer and Veerabhadrappa, 1965; Yoshida et al., 1977), scorpion proline oxidase activity was also stimulated by cytochrome c (Fig. 2.19). Maximal stimulation of the insect enzyme occurred at 1 μM cytochrome c (Brosemmer and Veerabhadrappa, 1965; Yoshida et al., 1977) while the scorpion enzyme required 1.6 to 2.0 μM concentration for maximal enhancement of activity (Fig. 2.19). The Km values calculated for cytochrome c in two experiments were 0.6 μM and 0.3 μM.

The activity of proline oxidase in heart and pedipalp muscles of the scorpion are given in Table 2.11. The activity of this enzyme could not be detected in hepatopancreas, alimentary canal, nervous system and telson.

**DISCUSSION**

The presence of the enzymes ornithine amino-transferase, Δ'-pyrroline-5-carboxylate reductase, Δ'-pyrroline-5-carboxylate dehydrogenase and proline oxidase
Effect of cytochrome c concentrations on proline oxidase activity in scorpion muscle. The proline oxidase activity (V) was assayed in the presence of different concentrations of cytochrome c (S) at a fixed concentration of L-proline (158 mM). S versus V plot is shown in Fig. A and double reciprocal plot in Fig. B.
Figure 2.19
Distribution of proline oxidase activity in scorpion tissues.

Values given are means ± standard deviations with ranges in parentheses.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>μmoles/hr/gm</th>
<th>μmoles/hr mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart (5)</td>
<td>8.4 ± 3.7</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(1.9 - 11.4)</td>
<td>(0.02 - 0.07)</td>
</tr>
<tr>
<td>Pedipalp muscles (7)</td>
<td>3.9 ± 0.9</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(2.9 - 5.5)</td>
<td>(0.02 - 0.05)</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>Telson</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>Alimentary canal</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>Nervous system</td>
<td>BLD</td>
<td>BLD</td>
</tr>
</tbody>
</table>

BLD : Below the level of detection.
suggest the ability of scorpion tissues to carry out the interconversions of ornithine, proline and glutamate. Of these enzymes, only ornithine aminotransferase is reversible, the other three being unidirectional enzymes (Herzfeld et al., 1977). The three unidirectional enzymes potentially orient the metabolic flow from ornithine to proline and glutamate on one hand and from proline to glutamate on the other. Labelled arginine or ornithine give rise to labelled proline and glutamate in animal tissues (Katunuma et al., 1965; Hill and Chambers, 1967; Mepham and Linzell, 1967; Verbeke et al., 1968; Reddy and Campbell, 1969b; Clark et al., 1975; Mezl and Knox, 1977; Yoshida et al., 1977). Similarly conversion of proline to glutamate has been demonstrated in mammalian (Holtzapple et al., 1973; Kowaloff et al., 1976) and insect (Sacktor, 1975) tissues.

The metabolic function of the reversible enzyme, ornithine aminotransferase, has been debated. Katunuma et al. (1965) proposed that it functions in ornithine catabolism. However, Herzfeld and Knox (1968) have opined that it serves primarily to provide ornithine for use in the urea cycle. Volpe et al. (1969) suggested that this enzyme probably exists in two isozymic forms, one functioning in the direction ornithine synthesis and the other in the direction of glutamate formation. The conversion of proline into
ornithine has been demonstrated in mammalian tissues (Smith et al., 1967). Although the equilibrium constant of ornithine aminotransferase is highly favourable for the formation of Δ^-pyrroline-5-carboxylate (Strecker, 1965), it has been argued that either compartmentation or coupling to other reactions might drive the reaction in the direction of ornithine formation (Smith et al., 1967). Conversion of glutamate into proline and citrulline has also been demonstrated in rat intestine (Windmueller and Spaeth, 1974, 1975, 1976), and the enzyme responsible for the reduction of glutamate to Δ^-pyrroline-5-carboxylate has also been elucidated recently in mammalian cells (Smith et al., 1980). While Shen and Strecker (1975) showed that glutamate was better than arginine as a precursor for proline, Smith and Phang (1979) concluded that ornithine is a more important source of proline formation than glutamate in mammalian cells. Taking the available evidence into account, McEwan et al. (1977) have concluded that ornithine aminotransferase is primarily involved in ornithine catabolism.

The scorpion tissues have the necessary compliment of enzymes (arginase, ornithine aminotransferase, Δ^-pyrroline-5-carboxylate reductase and Δ^-pyrroline-5-carboxylate dehydrogenase) to convert excess dietary arginine into proline and glutamate. In the arginine-proline pathway of scorpion
hepatopancreas, ornithine aminotransferase appears to be the rate limiting enzyme, while in the arginine glutamate pathway Δ'-pyrroline-5-carboxylate dehydrogenase is the rate limiting enzyme. Two enzymes, ornithine carbamoyltransferase and ornithine aminotransferase, compete for the ornithine formed in the arginase reaction. The high V\text{max} (80 μmoles/hr/gm tissue) and low Km for L-ornithine (0.6 and 1.2 mM) of ornithine carbamoyltransferase compared to those of ornithine aminotransferase (40 μmoles/hr/gm tissue, 2.0-5.8 mM) probably favour the metabolic conversion of ornithine to citrulline than to Δ'-pyrroline-5-carboxylate. However, a carbamoylphosphate synthetase is yet to be demonstrated in the scorpion and the role of citrulline (a metabolic dead-end product) in the physiology of the scorpion remains to be elucidated. Similarly Δ'-pyrroline-5-carboxylate dehydrogenase and Δ'-pyrroline-5-carboxylate reductase compete for the product of the ornithine aminotransferase reaction. While the Km of Δ'-pyrroline-5-carboxylate dehydrogenase (0.14-0.36 mM) for Δ'-pyrroline-5-carboxylate is only about half of that of Δ'-pyrroline-5-carboxylate reductase (0.53 mM), the V\text{max} of the latter enzyme is 6-12 times higher than that of the former (Tables 2.6 and 2.8). Hence, one is tempted to speculate that Δ'-pyrroline-5-carboxylate is more preferentially converted to proline than to glutamate in scorpion hepatopancreas.
These considerations, however, are by no means conclusive and are based on the assumption that factors like cellular compartmentation, concentrations of coenzymes and cosubstrates as well as other conditions in the intracellular environment are equally congenial for the catalytic function of the two competing enzymes. It has been shown that arginine catabolism is directed more towards proline formation than towards glutamate formation in Tetrahymena (Hill and Chambers, 1967), Liverfluke (Kurlec, 1975), silkworm fat body (Reddy and Campbell, 1969b) and mammary slices (Clark et al., 1975). However, in mammalian liver (Katunuma et al., 1965), mammary gland in vivo (Neal and Knox, 1977) and bacteria (Ramaley and Bernlohr, 1966) the pathway appears to be more directed towards glutamate synthesis.

One of the extra-ureagenic roles attributed to arginase in animals that lack a functional urea cycle is that it serves as a catabolic enzyme converting arginine to proline and glutamate in conjunction with ornithine aminotransferase, Δ'-pyrroline-5-carboxylate reductase and Δ'-pyrroline-5-carboxylate dehydrogenase. In the silkworm, which is deficient in urea cycle enzymes, arginase activity increases several fold during pupal-adult development to meet the proline requirements of the flight muscle mitochondria in the flying moth (Reddy and Campbell, 1969b). Similarly
in the mammary gland, which is devoid of a functional urea cycle, there is a coordinate increase in the activities of arginase, ornithine aminotransferase, \( \Delta^1 \)-pyrroline-5-carboxylate reductase and \( \Delta^1 \)-pyrroline-5-carboxylate dehydrogenase during lactation to cater to the elevated requirements of proline and glutamate in milk protein synthesis (Yip and Knox, 1972; Mezl and Knox, 1977; Clark et al., 1975). In the liverfluke, *Fasciola hepatica*, where arginase is the only enzyme of the urea cycle present (Janssens and Bryant, 1969; Kurulec, 1972), there is an active catabolic pathway converting arginine into proline and the parasite is benefitted by the regeneration of NAD in the \( \Delta^1 \)-pyrroline-5-carboxylate reductase reaction (Kurulec, 1975).

The scorpion also lacks a functional urea cycle (Chapter 1), but has the enzymic potentiality to catabolize excess dietary arginine to proline and glutamate. If the arginine catabolic path is active in the scorpion, the question arises regarding the importance of proline and glutamate in the physiology of scorpion and other arachnids. *Arachnid* hemolymph is very rich in proline and glutamate. In particular, the lycoid spiders are similar to insects in the relative abundance of proline (37-55% of the total amino acids) in their hemolymph (Punzo, 1982). Proline and glutamate together account for about 31% of the total hemolymph amino acids in the horseshoe crab *Limulus polyphemus*.
(Stevens et al., 1961) and 40% of the total hemolymph amino acids in the scorpion *Androctonus australis* (Briquet-Gregoire et al., 1963). Briquet-Gregoire et al. (1966) also reported that proline and glutamate are quantitatively the most predominant amino acids in the hemolymph of *Limulus polyphemus* and that there is more proline than glutamate in the blood, but more glutamate than proline in tissues like hepatopancreas and muscle. Furthermore, proline is the predominant amino acid in resilin and other cuticular proteins of arthropods (Anderson and Weis-Fogh, 1964; Karlson et al., 1969; Srivastav, 1971; Richards, 1978). Perhaps proline and glutamate are also put to use in other unidentified ways in the scorpion. It might appear paradoxical that arginine, which is an essential amino acid for arachnids (Rodriguez and Hampton, 1966), is used for the synthesis of non-essential amino acids like proline and glutamate. But it is not unreasonable to assume that the arginine catabolic pathway has evolved in animals as a mechanism of biochemical economy, wherein excess arginine is converted into proline and glutamate either to meet the requirements in some unknown physiological process or to provide for cellular energy.